ABSTRACT:

A series of 1'-mono-, di-, and trifluorinated analogs of propranolol and related steric congeners was prepared, and their metabolism was examined in recombinant-expressed CYP2D6. The structural changes in this series of compounds, principally added fluorines and methyl groups in the 1'-position of the N-isopropyl group, provided compounds that varied in pKₐ by more than 5 log units and also varied in lipophilicity and in steric size. Products of both aromatic hydroxylation and N-dealkylation were observed in the metabolic experiments. The regiochemistry of aromatic hydroxylation at the 4'- and 5'-positions was assigned based on high-pressure liquid chromatography, fluorescence, and mass spectral characteristics of the products and standards. Correlations of the metabolic kinetic parameters Kₘ and catalytic efficiency (kcat/Kₘ) with substituent parameters of the added groups showed that increased basicity (higher pKₐ values) was associated with increased enzyme affinity (low Kₘ values) and increased catalytic efficiency. More basic methyl-substituted compounds showed higher affinities for CYP2D6 than the structurally analogous less basic fluorinated congeners, indicating the decrease in affinity of the fluorinated compounds was not due to the size of the N-alkyl substituent. Correlations with log D reflected the degree of ionization and showed that the less lipophilic substrates (more basic compounds) had higher affinity for CYP2D6. These results are consistent with the proposal in the literature that ion pairing of the protonated amine of the substrate with Asp301 in the active site of CYP2D6 is very important to substrate affinity.

Aromatic ring hydroxylation and N-dealkylation are the major oxidative pathways of propranolol (P) metabolism (Fig. 1). In humans, the ring hydroxylation process produces regiosomeric phenolic metabolites, primarily via 4'-hydroxylation, with much less 5'-hydroxylation, smaller amounts of the 7-hydroxylation product, and very very small amounts of dihydroxylated products (Talaat and Nelson, 1988). A variety of evidence indicates that aromatic hydroxylation of P is catalyzed predominantly by CYP2D6. This process cosegregates with debrisoquine/sparteine polymorphism in vivo (Lennard et al., 1984; Raghuram et al., 1984). Significant inhibition of liver microsomal hydroxylation of P by quinidine occurs in vitro, and strong correlations with CYP2D6 content and debrisoquine-4-hydroxylation (Masubuchi et al., 1994) and 2-hydroxylation of desipramine have been observed (Yoshimoto et al., 1995). Inhibition by an antibody to rat CYP2D1 was also reported (Masubuchi et al., 1994). Based on the lack of complete inhibition by quinidine and a positive intercept in the linear correlation with CYP2D6 content, other P450 isozymes might contribute in a minor way to aromatic hydroxylation. Pharmacokinetic evidence for participation by an additional enzyme(s) has been obtained from incubations in human liver microsomes (Marathe et al., 1994).

Results using recombinant CYP2D6 in yeast cells showed formation of 4'-OHP and 5'-OHP and minor amounts of DIP (desisopropylpropranolol), with aromatic hydroxylation rates on the enantiomers of P exceeding those of N-dealkylation by ca. 10-fold (Bichara et al., 1996). When the enantiomers of P were studied separately in microsomes from recombinant CYP2D6 in human lymphoblastoid cells, the rate of 4'-hydroxylation exceeded the rate of N-dealkylation by 3- to 9-fold, but both metabolic processes were observed (Yoshimoto et al., 1995). Similar results have been obtained with recombinant enzymes and in human liver microsomes (Otton et al., 1990; Rowland et al., 1996).

Site-directed mutagenesis studies have identified Asp301 as being important for substrate binding and orientation in the active site of CYP2D6 and suggested that this negatively charged Asp residue interacts ionically with the protonated amine nitrogen of the substrate (Ellis et al., 1995). The binding pockets around the heme in the wild type and in these Asp301 mutants have been explored by migration of...
aryl groups from σ-bonded aryl-iron complexes formed from aryl-substituted diazenes (Mackman et al., 1996). Migration of large aryl substituents to the A-pyrole ring occurred in all of the mutants and the wild type, indicating that loss of activity in the Asp301 mutants is probably not due to major structural reorganization but to the loss of the ion-pairing interaction with the substrate (Mackman et al., 1996). Sequence alignments of CYP2D6 with CYP101 (P450cam) and CYP102 (P450bmt) place Asp301 within SRS-4 (Gotoh, 1992). Protein homology models of CYP2D6 based on the crystal structures for CYP101 and CYP102 place Asp301 in the I helix at an appropriate distance from the heme catalytic center to influence substrate binding (Koymans et al., 1993; Lewis et al., 1997).

Typical substrates for CYP2D6 are basic alicyclic amines, and often there is a flat hydrophobic region near the site(s) of oxidation. Pharmacophore models suggest the preferred sites of oxidation tend to be about 5 to 7 Å from the nitrogen (Koymans et al., 1992). NMR relaxation studies on codeine in the presence of CYP2D6, with its O-methyl group about 8 Å away from the basic nitrogen (de Groot et al., 1999a), suggest that it binds with the O-methyl group close to the heme iron (3.1 Å) (Modi et al., 1996). Imposing distance constraints, derived from the NMR studies on a protein homology model to generate a model of the codeine-CYP2D6 complex, positions the nitrogen atom of codeine close to Asp301 (Modi et al., 1996). Substrates with larger aryl or aralkyl groups, such as amitriptyline and imipramine, are oxidized primarily on the aromatic ring or adjacent to the ion-pairing interaction with the substrate (Mackman et al., 1996). Sequence alignments of CYP2D6 with CYP101 (P450cam) and CYP102 (P450bmt) place Asp301 within SRS-4 (Gotoh, 1992). Protein homology models of CYP2D6 based on the crystal structures for CYP101 and CYP102 place Asp301 in the I helix at an appropriate distance from the heme catalytic center to influence substrate binding (Koymans et al., 1993; Lewis et al., 1997).

Materials and Methods

Instrumentation. NMR Spectroscopy. NMR spectra were acquired on a Varian VXR 300 spectrometer (Varian, Inc., Palo Alto, CA) or a Bruker AF-300 spectrometer (Bruker Instruments, Inc., Billerica, MA). 1H and 13C NMR spectra were calibrated using neat trifluoroethanol (Aldrich, Milwaukee, WI) as an external reference, δ = −77.8 ppm relative to CFCl3 (Everett, 1995).

Mass Spectrometry. ESI mass spectra were obtained using a Finnigan LCQ quadrupole ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with a capacitive electrospray ion source modification (Wang and Hackett, 1998) of the Finnigan API interface. Collision-induced dissociation was carried out in the mass analyzer on an ion selected from the mass spectrum, using the He gas present in the trap. The samples, either synthetic standards or metabolic product mixtures in CH3OH, were infused directly via a syringe pump at a flow rate of 300 nL/min. The heated capillary was maintained at 160 °C and the source voltage at 1.7 kV.

Synthesis. Propranolol-Related Substrates and Standards. Propranolol enantiomers (25-P and 2R-P) were obtained from Sigma (St. Louis, MO). All other compounds were prepared in our laboratory. The fluorinated analogs PF, DFP, and TFP and the sterically hindered analogs iPMe and iBuMe (Beedle et al., 1989) were prepared as their diastereomers from 25-DIP (Cardillo et al., 1987). This amine was prepared from 25-1,2-epoxy-3-(1-naphthoxy)propane by a reaction with ammonia (Powell et al., 1980), followed by reductive amination (sodium cyanoborohydride) of mono-, di-, or trifluoroacetone, isopropyl methyl ketone, and tert-butyl methyl ketone, respectively. 1,1-Difluoroacetone was prepared by a reaction of 1,1-dichloroacetone with potassium bifluoride (Shapiro et al., 1973; Hudicky, 1976). Other ketones were obtained from Aldrich (Milwaukee, WI). Reductive amination (sodium cyanoborohydride) of trifluoroacetalddehyde mono methyl acetal (Aldrich) with 25-DIP gave the N-trifluoroethyl analog TFE (Weglicki et al., 1998). 4'-OH, 5'-OH, and 7'-OH standards were synthesized as previously reported (Oatis et al., 1981).

25-DIP. 1-Naphthol (480 mg, 3.33 mmol) and sodium hydride suspension (80% in oil, 130 mg, 4.3 mmol) were combined in dry DMF (10 mL). After stirring at room temperature for 25 min, 25-glycidyl tosylate (653 mg, 2.86 mmol) dissolved in dry DMF (5 mL) was added dropwise over 5 min. After stirring for 4 h at room temperature, the reaction mixture was partitioned between H2O (50 mL) and Et2O (50 mL). The aqueous layer was washed with more Et2O (50 mL). The combined Et2O extracts were washed with aqueous 1 N NaOH (20 mL) and H2O (10 mL), dried over MgSO4, and the solvent evaporated to afford the intermediate epoxide, which was used without further purification.

To the epoxide (527 mg, 2.64 mmol) was added a 2 M solution of NH3 in

![Figure 1: Major oxidative pathways of propranolol metabolism.](image)
methanol (20 mL, 40 mmol NH₄). The reaction mixture was covered tightly and stirred at room temperature. Progress of the reaction was monitored by TLC on silica using a mixture of 84% CH₂Cl₂, 12% EtOAc, 2% CH₂OH, and 2% triethylamine. At the end of 54 h, the remaining NH₄ and CH₂OH were evaporated to leave a tan solid. The crude product was partitioned between aqueous 2 N HCl (50 mL) and a mixture of Et₂O (100 mL) and EtOAc (50 mL). The aqueous layer was separated and made alkaline by addition of aqueous 6 N NaOH to bring the pH to 11. The alkaline aqueous solution was extracted with CH₂Cl₂ (4 × 50 mL). The combined organic extract was washed with H₂O (10 mL), dried over Na₂SO₄, and the solvent evaporated to yield 265 mg (46%) of 2R-DIP as a white solid.¹ H NMR (CDCl₃): δ 8.23 (1H, m, H-8'); 7.80 (1H, m, H-5'); 7.45 (3H, m, H-7', -6', -4'); 3.76 (1H, dd, H-3'); 6.83 (3H, m, H-2'); 4.16 (3H, m, H-3, -2); 3.02 (2H, m, H-1). ESIMS-MS [MH⁺] 218 → [C₉H₁₀O] + 183, [C₈H₇NO] 74.

2R-DIP. The 2R-enantiomer was prepared using 2R-glycidyl tosylate (649 mg, 2.85 mmol) and 1-naphthyl (482 mg, 3.3 mmol) by the procedure described above for the 2S-enantiomer to yield 236 mg (38%) of 2R-DIP as a white solid.¹ H NMR (CDCl₃): δ 8.23 (1H, m, H-8'); 7.80 (1H, m, H-5'); 7.45 (3H, m, H-7', -6', -4'); 3.76 (1H, dd, H-3'); 6.83 (3H, m, H-2'); 4.16 (3H, m, H-3, -2); 3.02 (2H, m, H-1). ESIMS-MS [MH⁺] 218 → [C₉H₁₀O] + 183, [C₈H₇NO] 74.

1°-FP. 2S-DIP (49 mg, 0.22 mmol) and sodium cyanoborohydride (57 mg, 0.99 mmol) were dissolved in CH₂OH (1 mL). Monofluoroacetone (100 mg, 1.5 mmol) and acetic acid (−20 μL) were added to the reaction mixture. It was capped tightly and stirred at 100°C for 2 h. Methylene chloride (4 mL) was added, and the solution was washed with saturated aqueous Na₂CO₃ solution (1 mL) and H₂O (1 mL). After evaporation of the organic solvent, the amine was purified by flash column chromatography, affording 18 mg (18%) of FP.¹ H NMR (CDCl₃): δ 8.25 (1H, m, H-8'); 7.81 (1H, m, H-5'); 7.48 (3H, m, H-7', -6', -4'); 7.39 (1H, dd, H-3'); 6.82 (1H, m, H-2'); 4.40 (2H, m, CH₂), 4.19 (3H, m, H-3, -2); 3.00 (3H, m, H-1, -2); 2.15 (3H, d, 2CH₃). FP-NMR (CDCl₃): δ = −224.3 (dt, J = 51 Hz, J = 19 Hz) and −224.5 (dt, J = 51 Hz, J = 19 Hz). ESIMS-MS [MH⁺] 278 → [C₉H₁₀O] + 183, [C₈H₇NO] 134.

1°-DIP. ² H NMR (CDCl₃): δ 8.24 (1H, m, H-8'); 7.81 (1H, m, H-5'); 7.47 (3H, m, H-7', -6', -4'); 7.39 (1H, dd, H-3'); 6.82 (1H, m, H-2'); 5.69 (1H, tm, CH), 4.18 (3H, m, H-3, -2); 3.07 (3H, m, H-1, -2'); 1.19 (3H, d, 2CH₃). PF-NMR (CDCl₃): δ = −124.29 and −124.43 (dd, J = 300 Hz, J = 60 Hz, J = 11 Hz) and −126.5 and −126.9 (dd, J = 300 Hz, J = 60 Hz, J = 12 Hz). ESIMS-MS [MH⁺] 296 → [C₉H₁₂O] + 183, [C₈H₈NO] 152.

2S°-F, 1°-DIP. ³ H NMR (CDCl₃): δ 8.22 (1H, m, H-8'); 7.81 (1H, m, H-5'); 7.48 (3H, m, H-7', -6', -4'); 7.39 (1H, dd, H-3'); 6.82 (1H, m, H-2'); 4.19 (3H, m, H-3, -2); 3.10 (3H, m, H-1, -2'); 1.31 (3H, d, 2CH₃). ¹F NMR (CDCl₃): δ = −77.14 (J = 7 Hz) and −77.34 (J = 7 Hz). ESIMS-MS [MH⁺] 314 → [C₉H₁₂O] + 183, [C₈H₈NO] 170.

2S°-I°-F. ¹H NMR (CDCl₃): δ 8.22 (1H, m, H-8'); 7.81 (1H, m, H-5'); 7.48 (3H, m, H-7', -6', -4'); 7.39 (1H, dd, H-3'); 6.82 (1H, m, H-2'); 4.19 (3H, m, H-3, -2); 3.10 (3H, m, H-1, -2'); 1.31 (3H, d, 2CH₃). ¹F NMR (CDCl₃): δ = −77.14 (J = 7 Hz) and −77.34 (J = 7 Hz). ESIMS-MS [MH⁺] 314 → [C₉H₁₂O] + 183, [C₈H₈NO] 170.

2S°-I°-F. ²H NMR (CDCl₃): δ 8.22 (1H, m, H-8'); 7.81 (1H, m, H-5'); 7.48 (3H, m, H-7', -6', -4'); 7.39 (1H, dd, H-3'); 6.82 (1H, m, H-2'); 4.19 (3H, m, H-3, -2); 3.10 (3H, m, H-1, -2'); 1.31 (3H, d, 2CH₃). ¹F NMR (CDCl₃): δ = −77.14 (J = 7 Hz) and −77.34 (J = 7 Hz). ESIMS-MS [MH⁺] 314 → [C₉H₁₂O] + 183, [C₈H₈NO] 170.

Deshydroxyseliproprioanal (desOHDP). Preparation of this primary amine was modified from the reported procedure (Glenon et al., 1989). 1-Naphthol (4.0 g, 28 mmol) and sodium hydride (80% in oil, 1.5 g, 50 mmol) were combined in DMF (25 mL) under a dry argon atmosphere. After 20 min at room temperature, N-(3-bromopropyl)phenylphthalamide (5.0 g, 18.6 mmol) was added, and the reaction mixture heated to reflux. After 15 h, the DMF was evaporated, and the remaining residue was partitioned between 25 mL H₂O and 50 mL Et₂O. The aqueous phase was then extracted with CH₂Cl₂ (3 × 5 mL). The combined CH₂Cl₂ extracts were washed with H₂O, dried over MgSO₄, and solvent evaporated to yield a white crystalline solid, 285 mg (46%) of the naphthoxypropylphthalamide. Hydrazine (4.0 g, 125 mmol) in ethanol (15 mL) was added dropwise to a solution of the phthalamide (430 mg, 13 mmol) in ethanol (10 mL). The reaction mixture was then heated to reflux for 2 h. At the end of this time the precipitate was removed by filtration and extracted with hot ethanol (40 mL). The ethanol was evaporated from the filtrate and the
residue was dissolved in 20 ml of CH₂Cl₂ and extracted with aqueous 1 N HCl (40 ml, and then 2 × 20 ml). After the combined aqueous layers were adjusted to pH 11 by addition of aqueous 6 N NaOH, the amine was extracted into CH₂Cl₂. The combined CH₂Cl₂ extracts were washed with H₂O, dried over Na₂SO₄, and evaporated affording 98 mg (38%) of desOHDIP. ¹H NMR (CDCl₃): δ 8.25 (1H, m, H-8); 7.89 (1H, m, H-5); 7.41 (4H, m, H-7, -6, -4, -3); 6.79 (1H, d, H-2); 4.19 (2H, t, H-3); 3.35 (2H, N-H), 3.07 (2H, t, H-1); 2.16 (2H, t, H-2); ESI-MS/MS [MH⁺] 202 → [C₆H₄O⁺] 185.  

**desOHP.** Primary amine desOHDIP (402 mg, 2.0 mmol) and acetone (544 mg, 9.4 mmol) were dissolved in benzene (5 ml), and the mixture flushed with dry argon and stirred at room temperature for 46 h. At the end of this time, the benzene was evaporated and the imine reduced. It was dissolved in CH₃OH (10 ml), and sodium cyanoborohydride (500 mg, 8.0 mmol) and acetic acid (100 μl) were added. After the mixture was stirred at room temperature for 8 h, the mixture was partitioned between CH₂Cl₂ (20 ml) and aqueous 1 N NaOH (20 ml). The aqueous layer was extracted with additional CH₂Cl₂ (2 × 10 ml), and the combined CH₂Cl₂ layers were washed with H₂O (5 ml), dried over Na₂SO₄, and the solvent evaporated. The resulting oil was purified on a silica flash column and eluted with stepwise changes in solvent from CH₂Cl₂ to 50% EtOAc in CH₂Cl₂, affording 250 mg (51%) of desOHP. ¹H NMR (CDCl₃): δ 8.38 (1H, m, H-8); 7.85 (1H, m, H-5); 7.48 (4H, m, H-7, -6, -4, -3); 6.85 (1H, d, H-2); 4.25 (2H, t, H-3); 3.21 (1H, qq, H-2); 3.05 (2H, t, H-1); 2.11 (2H, tt, H-2); 1.28 (3H, d, 2×CH₃). ESI-MS/MS [MH⁺] 214 → [C₆H₄O⁺] 185, [C₆H₅N⁻] 100.  

**desOHTFP.** ¹H NMR (CDCl₃): δ 8.80 (1H, m, H-8); 7.85 (1H, m, H-5); 7.48 (4H, m, H-7, -6, -4, -3); 6.85 (1H, d, H-2); 4.25 (2H, t, H-3); 3.21 (1H, qq, H-2); 3.05 (2H, t, H-1); 2.11 (2H, tt, H-2); 1.28 (3H, d, 2×CH₃). ESI-MS/MS [MH⁺] 298 → [C₆H₄O⁺] 185, [CH₃CH₂NO⁻] 154.  

**4'-Hydroxydeshydroxypropranolol (4'-OHdesOHP).** 4-Methoxy-1-naphthol (843 mg, 4.8 mmol) was dissolved in DMF (8 ml, dried over KOH). Sodium hydride (80% in oil, 500 mg, 16.6 mmol) was rinsed with petroleum ether and then transferred with 6 ml of dry DMF into the reaction vessel. After stirring for 20 min under a dry argon atmosphere, a solution of N-(3-bromopropyl)phthalimide (1.39 g, 5.2 mmol) in 10 ml of dry DMF was added and the reaction mixture and heated to reflux. The mixture was heated at reflux for 6 h, while monitoring the progress of the reaction by TLC (CH₂Cl₂). The solvent was evaporated, and the remaining residue was partitioned between 50 ml H₂O and 50 ml CH₂Cl₂. The aqueous phase was then extracted with CH₂Cl₂ (25 ml). The combined CH₂Cl₂ extracts were washed with H₂O, dried over MgSO₄, and the solvent evaporated to yield the phthalimide intermediate as a brown solid, 1.79 g (~100%).  

The crude phthalimide (1.79 g, 5.0 mmol) was dissolved in ethanol (60 ml), and a solution of hydrazine (1 ml, 30 mmol) in ethanol (15 ml) was added dropwise. After the addition, the reaction mixture was heated to reflux for 3 h. Allowing the solution to cool slightly resulted in a large amount of a precipitate. Filtering the solution afforded 1.10 g of a brown oil (96%) of crude 3-(4-methoxy-1-naphthoxy)propylamine, which was used without further purification.  

**Propanolol O-Methyl Ether (MeO-P).** Propanolol HCl (100 mg, 0.34 mmol), triethylamine (200 μl, 1.4 mmol), and di-tert-butyl dicarbonate (73.8 mg, 0.34 mmol) were dissolved in 10 ml of CH₂Cl₂. The mixture was heated to reflux for 2 h. After evaporating the solvent and triethylamine, the residue was dissolved in CH₂Cl₂ (20 ml), washed with 0.5 N HCl (2 × 10 ml) and then H₂O (10 ml), dried over Na₂SO₄, and the solvent was evaporated. The remaining oil, the N-t-butyl carbamate of P (110 mg, 0.28 mmol), was dissolved in tetrahydrofuran (10 ml). Powdered KOH (78 mg, 1.34 mmol) and CH₂Cl₂ (200 μl, 3.2 mmol) were added. The mixture was sealed (septum) and stirred at room temperature for 15 h. Water (10 ml) and CH₂Cl₂ (10 ml) were added, and the CH₂Cl₂ layer was removed, washed with H₂O (5 ml), dried over Na₂SO₄, and the solvent evaporated. The remaining O-methyl ether carbamate was dissolved in a mixture of concentration HCl (1 ml) and EtOAc (4 ml) and then stirred at room temperature for 30 min. After making the solution alkaline by addition of aqueous 5 N NaOH, the EtOAc layer was removed, washed with H₂O, dried over Na₂SO₄, and the solvent was evaporated to yield MeO-P, 75 mg (80%). ¹H NMR (CDCl₃): δ 8.25 (1H, m, H-8); 7.78 (1H, m, H-5); 7.40 (4H, m, H-7, -6, -4, -3); 6.81 (1H, d, H-2); 4.20 (2H, m, H-3); 3.89 (1H, m, H-2); 3.58 (3H, s, OCH₃); 2.89 (3H, m, H-1, -2); 1.10 (6H, d, 2×CH₃). ESI-MS/MS [MH⁺] 314 → [C₇H₁₀O₄][⁺] 199, [C₄H₄NO⁺][⁺] 170.
Results

Fluorinated and Steric Analogs of P and Standards. The propranolol-related compounds prepared for the metabolic studies are listed in Table 1. These include the 1'-mono-, di-, and tri-fluorinated derivatives (FP, DFP, TFP) and steric congeners with two and three -methyl groups (iPrMe, tBuMe). Reductive amination of the corresponding ketones using a single enantiomer of DIP and sodium cyanoborohydride afforded the desired P analogs. TFE was prepared by a similar procedure, reductive amination of trifluoroacetalddehyde mono methyl acetal. Examples of these procedures are given under Materials and Methods. To examine the metabolism of a single enantiomer of TFP, its 2'S,1'R-enantiomer was prepared from 2S-1-naphthoxy-2,3-epoxypropane (Klunder et al., 1986) and 2R-1,1,1-trifluoro-2-propylamine (Soloshonok and Ono, 1997), using alumina as a catalyst (Posner and Rogers, 1977a,b). Deshydroxy analogs desOHP and desOHTFP were also prepared by reductive amination of acetone and trifluoroacetone, respectively, using desOHDIP and sodium cyanoborohydride. Standards of 4'-hydroxylated metabolites of desOHP and TFP (4'OH-desOHP and 4'OHTFP) were prepared to aid in the identification of metabolites, and MeO-P was prepared as an analytical standard. Methods for their preparation are also given under Materials and Methods.

Basicity, Partition and Distribution Coefficients, and Steric Parameters. The pKₐ values of the compounds in the series and data on their partition characteristics appear in Table 1. Octanol-water partition coefficients (log P) and distribution coefficients (log D) at physiological pH were determined. To provide an indication of the steric effect of adding fluorines and methyl groups, steric parameters from the literature are tabulated in Table 2.

pKₐ Values. The sequential addition of fluorines to the N-isopropyl substituent causes a regular decrease in the pKₐ of the amine by 1.6 to 1.8 pKₐ units per fluorine (Table 1), whereas the pKₐ values of our nonfluorinated congeners iPrMe and tBuMe are similar to that of P.
amine being protonated to less than 1% at physiological pH) show log D values equal to their log P values, as expected.

**Steric Parameters.** A measure of the relative size of the substituents is given by the parameters in Table 3. The Taft steric parameter $E_v$ (Taft, 1956) and the Charton van der Waals radius $r_v$ (Charton $v$ parameter modified by the addition of the van der Waals radius of the hydrogen atom) (Charton, 1975) is given for each of the substituents. Although fluorine is sometimes considered to be isosteric with hydrogen, it is somewhat larger. The generally accepted value for the van der Waals radius of fluorine is 1.47 Å, closer to oxygen at 1.52 Å than to hydrogen at 1.20 Å (Smart, 1994), and the $sp^3$ C-F bond is longer than the $sp^3$ C-H bond (1.40 Å versus 1.09 Å) (March, 1992). The addition of multiple fluorines can cause a significant increase in size of an alkyl group. These values show that the sizes of the isopropyl and $t$-butyl substituents in iPrMe and tBuMe bracket those of the trifluoromethyl group in TFP, being slightly smaller and slightly larger, respectively.

**Identities of Metabolites Formed by Incubation with CYP2D6.** Incubations of all the substrates in CYP2D6 resulted in formation of two metabolites with retention times shorter than that of the parent drug and small amounts of $N$-dealkylation product. Representative chromatograms showing the retention times of ring-hydroxylated metabolites from DFP and TFP are presented in Figs. 2 and 3. The collision-induced dissociation-mass spectra (also in Figs. 2 and 3) of the indicated peaks all clearly show $[M + H]^+$ ions at $m/z$ values corresponding to addition of an oxygen atom and fragment ions with correct $m/z$ values for the hydroxylated ring-containing ion (usually the base peak; $m/z = 199$) and for the side chain ion, which had an $m/z$ value corresponding to no addition of oxygen (Upthagrove et al., 1999).

We were unable to determine the position of hydroxylation on the aromatic ring from the ESI mass spectra alone, so the fluorescence excitation and emission spectra of the ring-hydroxylated metabolites were examined. As shown in Fig. 4, the $4'$-hydroxylated metabolites of P and TFP have nearly identical fluorescence spectra. The wavelength maxima of the fluorescence excitation and emission spectra of metabolite standards for $4'$-, $5'$-, and $7'$-OH (Fig. 4), however, vary depending on the location of the hydroxyl group. In addition, the number of distinct maxima in the excitation spectra depends on the location of the hydroxyl group. Thus, distinguishing between the ring-hydroxylated regioisomers was based on their characteristic fluorescence excitation and emission spectra. Fractions of HPLC eluent containing the unknown metabolites from each of the substrates were collected and their fluorescence excitation and emission spectra measured. For each of the substrates, the unknown metabolite with the

<table>
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<th>Substrate</th>
<th>$4'$-OH Product</th>
<th>$5'$-OH Product</th>
<th>N-Dealkylation Product</th>
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<td>2S-P</td>
<td>2.5 ± 0.4</td>
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<td>20.6</td>
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<tr>
<td>2R-P</td>
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<td>2S-FF</td>
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<tr>
<td>2S-DFP</td>
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<td>18.4 ± 0.3</td>
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<td>3.4 ± 0.7</td>
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<tr>
<td>2S-TFP</td>
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<td>75.2 ± 6.5</td>
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<tr>
<td>2S,4R-TFP</td>
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<td>27.0 ± 1.0</td>
<td>0.6</td>
<td>38.6 ± 0.8</td>
</tr>
<tr>
<td>2R-TFP</td>
<td>32.5 ± 5.5</td>
<td>34.6 ± 1.7</td>
<td>1.1</td>
<td>33.6 ± 2.3</td>
</tr>
<tr>
<td>iPrMe</td>
<td>0.32 ± 0.07</td>
<td>19.8 ± 0.6</td>
<td>61.9</td>
<td>0.44 ± 0.18</td>
</tr>
<tr>
<td>tBuMe</td>
<td>0.25 ± 0.06</td>
<td>7.1 ± 0.2</td>
<td>28.4</td>
<td>0.17 ± 0.05</td>
</tr>
<tr>
<td>TFP</td>
<td>46.9 ± 5.3</td>
<td>25.5 ± 1.1</td>
<td>0.5</td>
<td>41.4 ± 2.4</td>
</tr>
<tr>
<td>desOHP</td>
<td>0.16 ± 0.11</td>
<td>22.0 ± 1.3</td>
<td>139</td>
<td>0.22 ± 0.12</td>
</tr>
<tr>
<td>desOHTFP</td>
<td>9.49 ± 1.20</td>
<td>17.9 ± 0.6</td>
<td>1.9</td>
<td>7.3 ± 1.5</td>
</tr>
</tbody>
</table>
shorter retention time had fluorescence spectra similar to 5'-OHP (compare Figs. 2 and 3 with Fig. 4), whereas the unknown metabolite with the longer retention time had fluorescence spectra similar to 4'-OHP. The elution order of the 4'- and 5'-hydroxylated metabolites is also consistent with the elution order of 5'- and 4'-OHP standards under the chromatographic conditions. Taken together, the mass spectra, fluorescence spectra, and relative retention times of the observed metabolite peaks suggest that in incubations with CYP2D6, all of the substrates studied gave products of 4'- and 5'-hydroxylation and DIP.

**Metabolite Quantitation.** The amounts of metabolites for which standards were available were calculated based on standard curves, which were linear in the range used. Comparison of the slopes of the standard curves indicates that 4'-OHP, 4'-OHTFP, and 4'-hydroxydeshydroxypropranolol have nearly identical responses under our fluorescence detection conditions. On this basis, we assumed that the 4'-hydroxylated metabolites from the other substrates would have similar responses, and the amounts of these metabolites could be estimated based on comparison to standard curves for the synthetic metabolite standards. Similarly, the amounts of the 5'-hydroxylated metabolites were estimated based on standard curves for 5'-OHP.

**Kinetic Parameters.** The $K_m$ and $k_{cat}$ values, determined from quantitation of each of the metabolites formed from incubations of
increasing substrate concentrations with CYP2D6 are summarized in Tables 3 and 4. There are marked changes in $K_m$ and $k_{cat}/K_m$ over the series. In all cases, ring hydroxylation is preferred over N-dealkylation. As the number of fluorines increased from zero to three, the $K_m$ increased in a regular manner. The TFP and TFE analogs exhibited at least 10-fold higher $K_m$ values than P in our system. Only a small difference is observed between the 2R-P and 2S-P. Likewise, the TFP diastereomers show relatively little stereoselectivity in this process.

Substituting larger nonfluorinated alkyl groups (iPrMe and tBuMe) resulted in $K_m$ values even lower than that for P. Both trifluorinated and nonfluorinated analogs without the side chain hydroxyl group resulted in lower $K_m$ values and higher $k_{cat}/K_m$ values compared with TFP and P, respectively.

**Correlations with Physicochemical Parameters.** To obtain more quantitative information about the changes in the CYP2D6-catalyzed metabolism of these analogs, physicochemical parameters pKₐ and log D (Table 1) were correlated with the metabolic kinetic parameters (Table 3). The $K_m$ values cover a 370-fold range, and the summed $k_{cat}$ terms vary only about 5-fold across the series. In Fig. 5, separate plots of log $K_m$ and log catalytic efficiency versus pKₐ of all the analogs are...
shown. There is a strong negative correlation of log $K_m$ with $pK_a$ for members of this series ($r^2 = 0.80$). Conversely, the log $k_{cat}/K_m$ is correlated positively and strongly with $pK_a$ ($r^2 = 0.88$). The more basic compounds have higher affinities (lower $K_m$ values) and higher catalytic efficiencies.

The slopes of the correlations of log $K_m$ and log $k_{cat}/K_m$ with log $D$ (Fig. 6) are opposite to those observed for the correlations with $pK_a$. The more lipophilic compounds have lower affinities and lower catalytic efficiencies. This is expected because an increasing proportion of ionized species at physiologic pH occurs with increasing $pK_a$, leading to a decrease in log $D$. There is significantly greater scatter in the correlations with log $D$ ($r^2 = 0.35$ and 0.43, respectively) compared with those with $pK_a$.

Discussion

Physicochemical Parameters. The range in $pK_a$ values (Table 1) among the fluorinated P analogs, about 5 $pK_a$ units, is expected. A similar wide range of changes have been observed in series of $\beta$-mono-, di-, and trifluorinated ethylamines (e.g., two series of $\beta$-fluoroethyl-substituted normeperidine and normetazocine analogs) in which basicity ranges from $pK_a$ values higher than 9.0 to 3.0 and to 4.5, respectively (Reifenrath et al., 1980). In the simple $\beta$-mono-, di-,
values (Table 1), and the order of increasing log D values is not the weight occurs with fluorination. Although a significant increase in molecular
trifluorinated primary aliphatic alcohol and its nonfluorinated ho-
molog (Muller, 1986), although a significant increase in molecular

Kluger and Hunt, 1984). Similarly, almost no change in log P is observed between a

Log Km = −0.34 pK_a + 2.95; r^2 = 0.80 (p < 0.05). Log k_cat/K_m = 0.30 pK_a −
1.16; r^2 = 0.88 (p < 0.05).

and trifluorinated ethylamines the range is similar, ranging from pK_a 10.7 to 5.4 (Kluger and Hunt, 1984).

These variable effects of fluorines on lipophilicity are consistent
and trifluoromethyl group (Table 2); substrates with the nonfluori-

1995). Similarly, almost no change in log P is observed between a

These compounds exhibit a larger range of log D values than log P
values (Table 1), and the order of increasing log D values is not the

same as the order of increasing log P values. These changes are in
large measure a function of their pK_a values. Not unexpectedly,
degree of protonation plays a very large role in determining log D
values, with the more basic compounds that are protonated to a greater
extent at pH 7.4 exhibiting the lower values.

Metabolism. We observed similar catalytic efficiencies for metab-

log D at pH 7.4

and trifluoromethyl group (Table 2); substrates with the nonfluori-

These compounds exhibit a larger range of log D values than log P
values (Table 1), and the order of increasing log D values is not the

the 2-position of the side chain to reduce the number of diastereomers in
the incubations. When diastereomeric TFP analogs with S and R
absolute stereochemistry at the 2-position and the single enantiomer
25,4R-TPF were examined, little difference was observed among
them. Inspection of Fig. 5 and Table 3 shows that the stereoselectivity
of metabolism of these compounds is small compared with the effects
of the fluorines. Thus, more extensive investigation of the effect of the
absolute stereochemistry seems unwarranted at this time.

The increase in steric bulk introduced with the added fluorines and
larger alkyl groups is not detrimental to metabolism by CYP2D6.

Although the Taft E_f and Charton van der Waals radius (r_v) values for
the t-butyl and isopropyl groups indicate that they are similar in size
to the trifluoromethyl group (Table 2); substrates with the nonfluori-
nated substituents are more rapidly metabolized, whereas the substrates
with similar sized fluorinated N-substituents are turned over more
slowly.

The metabolic effects we observed with changes in structure are
primarily the result of changes in substrate affinity, producing large
changes in the K_m term and much smaller changes in the k_cat term. The
K_m term dominates the correlations with log catalytic efficiency since
K_m ranges over a 370-fold range (2.5 log units), whereas the changes
in the k_cat term vary only about 5-fold (0.7 log units) (Table 4). The changes in k_cat/K_m vary about 190-fold (2.3 log units), only a slightly
smaller range than in the log K_m term in Figs. 5 and 6.

A limited range of k_cat terms is consistent with the high degree of
structural similarity of these substrates and with aromatic hydroxy-
lation of a common 1-oxy-substituted naphthalene ring system being the
major metabolic process. A limited range of k_cat terms might arise due
to significant contribution(s) of one or more of the kinetic steps
associated with P450 oxidation (e.g., substrate binding, ferric iron
reduction, oxygen binding to P450 ferrous iron, addition of the second
electron, and rearrangement to the final active oxygen species that
precede substrate oxidation). The possibility that slow product release
of the structurally very similar products is a significant kinetic deter-
ninant seems less likely, as this has not been previously observed in
extensive studies of P metabolism catalyzed by CYP2D6, CYP1A2,
or other P450 isozymes. In the CYP2 family of isozymes, slow
product release has been reported only for products from small mol-
ecule substrates of CYP2E1 (Bell and Guengerich, 1997).

The catalytic efficiency term (k_cat/K_m) provides little mechanistic
biochemical information, but it provides an excellent comparison of
expected relative metabolic rates of these structurally related sub-
strates under nonsaturating conditions. In the correlations with log
pK_a and log D, only small changes in the correlation coefficients of
log k_cat/K_m versus log K_m plots are observed (slight improvement),
both in 2D6 and in 1A2 (Upthagrove and Nelson, 2001).
2D6 METABOLISM OF FLUORINATED PROPRANOLOL ANALOGS

All of our data are consistent with the generally accepted model for binding of CYP2D6 substrates via formation of an ion pair of the protonated amine with the carboxylate anion of Asp301 in the enzyme active site and subsequent oxidation at a distant site in the molecule. Obviously, our substrates do not bind exclusively in this orientation since N-dealkylation occurs, requiring the nitrogen to be closer to the oxidation site. Because aromatic hydroxylation is the predominant pathway, by a factor of at least 5-fold for each of the substrates (Table 5), the models appear to be satisfactory constructs to describe the predominant mode of substrate binding to CYP2D6.

Although sequentially added fluorines reduce the basicity of the P analogs by up to 5 pK_a units, all of the compounds would be expected to participate to some degree in an ion-pairing with a carboxylate anion. The data clearly show that increasing lipophilicity is associated with increased affinity and catalytic efficiency by CYP2D6 when the pK_a values are similar. Our results showing better correlation of enzyme affinity and catalytic efficiency with pK_a alone than with log D alone suggest that greater emphasis should be placed on the pK_a values, but taken together they provide a more detailed description of substrate characteristics associated with metabolism by CYP2D6.

In summary, for this series of compounds, the changes in pK_a have the predominant effects on substrate binding. More basic amines are associated with higher affinities (lower K_m values) and higher catalytic efficiencies (k_cat/K_m) for CYP2D6. The steric effects of the N-substituent in the series are not detrimental to either the enzyme affinity (K_m) or catalysis (k_cat).